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Map of Genes for Carotenoid and Bacteriochlorophyll Biosynthesis in *Rhodopseudomonas capsulata*

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The recently discovered gene transfer system of *Rhodopseudomonas capsulata* was used to construct a genetic map of a region concerned with bacteriochlorophyll and carotenoid production. Mutants blocked in the biosynthesis of these compounds were isolated, and each was characterized on the basis of pigments accumulated during growth under low pO_2. One-point, two-point, three-point, and ratio test crosses were performed between various mutant strains, and the results were amenable to conventional genetic analyses. A mapping function was found that related cotransfer frequency to map distance. Seven clusters of mutations, five affecting carotenoid and two affecting bacteriochlorophyll biosynthesis, were arranged in one linkage group. Each cluster of mutations is thought to represent a gene. The length of the mapped region is estimated to be less than 1% of the genome. Cotransfer is observed between markers separated by about 5 to 10 genes.

The intracellular membranes of nonsulfur, purple photosynthetic bacteria are exceptionally well suited for the study of the regulation of membrane formation and differentiation. The synthesis of these membranes is dramatically influenced by O_2 tension and light intensity (1, 11) and, since the membranes are easily separable from other cellular components (3), qualitative and quantitative changes in their composition may readily be observed in response to regulatory stimuli. Furthermore, many species of *Rhodospirillaceae* grow equally well by either respiration or photosynthesis; thus, mutants devoid of functional photosynthetic membranes may easily be propagated for study. The most readily assayed components of the intracellular membranes are the photopigments bacteriochlorophyll (BChl) and carotenoids and, since BChl is also essential for the photochemical functions of the membranes, the regulation of the synthesis of these compounds has been the subject of many investigations (1, 8).

A serious limitation to the potential of this photosynthetic membrane model system has been the lack of a supporting experimental genetic system. We recently discovered a gene transfer process occurring in *Rhodopseudomonas capsulata* (10). The transfer mechanism resembles generalized transduction in that samples of donor deoxyribonucleic acid (DNA) are transmitted via nuclease-resistant particles to recipient cells, but no viral activities have been found associated with the particles themselves. In this report we demonstrate that this gene tranfer system can be used to construct genetic maps, and we present a map of a region concerned with BChl and carotenoid biosynthesis.

MATERIALS AND METHODS

Bacterial strains. The strains of *R. capsulata* used are described in Table 1. Mutants with altered pigments were generally found by inspection after nitrosoguanidine mutagenesis, although a few strains arose from spontaneous or ultraviolet-induced mutations. The symbol *crt* has been chosen to designate those loci involved in carotenoid biosynthesis. Mutants with blocks at various points in the carotenoid biosynthetic pathway have been separated into different classes on the basis of the color of colonies formed during aerobic (dark) incubation. Blue-green mutants accumulate no carotenoids with absorption in the visible region of the spectrum (Crt^-) and form dull gray colonies. (The name "blue-green" is only descriptive of the colonies formed under photosynthetic conditions, but we retain this nomenclature because it is so well established in the literature.) The mutants that we call "yellow" form brownish yellow colonies under aerobic (dark) conditions, whereas wild-type colonies are red under the same conditions. Colonies resulting from photosynthetic growth of yellow mutants are indistinguishable from the wild type. This phenotype appears to be the same as that termed "brown" by Griffiths and Stanier (4) but, since "brown" has also been applied to an entirely different phenotype by Segen and Gibson (12), we have chosen "yellow." In favor of this choice is the observation that the carotenoids accumulated by these mutants during aerobic (dark) growth spectrally resemble sphereoidene,

TABLE 1. *Bacterial strains*

Strain[a]	Genotype[b]	Remarks[c]	Strain[a]	Genotype[b]	Remarks[c]
B301	*crtA301, rif-10*	Yellow, Rif^R	W5	*crtE5*	Blue-green
BB101	*rif-10*	Rif^R	W6	*crtE6*	Blue-green
BW5	*crtE5, rif-10*	Blue-green, Rif^R	W7	*crtE7*	Blue-green
BW6	*crtE6, rif-10*	Blue-green, Rif^R	W8	*crtE8*	Blue-green
BW7	*crtE7, rif-10*	Blue-green, Rif^R	W9	*crtE9*	Blue-green
BY161	*crtB16, rif-10, asr-1, aer-103r16*	Blue-green, As^R, Rif^R, Aer$^+$	Y16	*crtB16, asr-1, aer-103r16*	Blue-green
BY392	*crtD39, str-2, rif-10*	Green, Str^R, Rif^R	Y34	*bchA34, str-2, trpA20*	PS$^-$, Trp$^-$, Str^R, P670
BY612	*crtC61, str-2, rif-10*	Green, Str^R, Rif^R	Y39	*crtD39, trpA20 str-2*	Green, Trp$^-$, Str^R
BY711	*crtA71, str-2, rif-10*	Yellow, Str^R, Rif^R	Y59	*crtC59, trpA20, str-2*	Green, Trp$^-$, Str^R
BY761	*crtC76, str-2, rif-10*	Green, Str^R, Rif^R	Y62	*bchA62, trpA20, str-2*	PS$^-$, Trp$^-$, Str^R, P670
BY771	*crtE77, str-2, rif-10*	Blue-green, Str^R, Rif^R	Y68	*crtC68, trpA20, str-2*	Green, Trp$^-$, Str^R
BY3612	*crtD361, str-2, rif-10*	Green, Str^R, Rif^R	Y71	*crtA71, str-2*	Yellow, Str^R
HH901	*crtA901, rif-9*	Yellow, Rif^R	Y77	*crtE77, str-2*	Blue-green, Str^R
HH902	*crtA902, rif-9*	Yellow, Rif^R	Y80	*bchB80, str-2*	PS$^-$, Str^R, P630
M112	*crtC112, rif-9*	Green, Rif^R	Y83	*crtD83, str-2*	Green, Str^R
MB1048	*crtB348, str-2*	Blue-green, Str^R	Y87	*crtA87, str-2*	Yellow, Str^R
R114	*crtC114, asr-1, aer-25r114*	Green, As^R, Aer$^+$	Y88	*crtC88, str-2*	Green, Str^R
			Y90	*crtE90, str-2*	Blue-green, Str^R
SB3	*crtA203, rif-10*	Yellow, Rif^R	Y92	*bchA92, str-2*	PS$^-$, Str^R, P670
SB7	*crtA207, rif-10*	Yellow, Rif^R	Y121	*crtE6, bchA121*	PS$^-$, POK^R, P670
SB9	*crtC209, rif-9*	Green, Rif^R	Y122	*crtE6, bchA122*	PS$^-$, POK^R, P670
SB21	*bchA221, rif-10*	PS$^-$, P670	Y125	*crtD125, str-2*	Green, Str^R
SB23	*crtD223, rif-10*	Green, Rif^R	Y126	*crtC126, str-2*	Green, Str^R
SB35	*crtA235, rif-10*	Yellow, Rif^R	Y127	*crtD127, str-2*	Green, Str^R
SB36	*crtC236, rif-10*	Green, Rif^R	Y129	*crtC129, str-2*	Green, Str^R
SB37	*crtD237, rif-10*	Green, Rif^R	Y150	*crtD150, str-2*	Green, Str^R
SB38	*crtD238, rif-10*	Green, Rif^R	Y165	*crtB4, bch-165*	PS$^-$, POK^R, P670
SB1003	*rif-10*	Rif^R	Y167	*crtB4, bch-167*	PS$^-$, POK^R, P670
W4	*crtB4*	Blue-green	Y361	*crtD361, trpA20, str-2*	Green, Trp$^-$, Str^R

[a] Strains W4, W5, W6, W7, W8, and W9 were obtained from P. Weaver; all others were isolated or constructed in our laboratory.

[b] Genetic symbols, traits affected: *aer*, aerobic growth; *asr*, arsenate sensitivity; *bch*, bacteriochlorophyll synthesis; *crt*, carotenoid synthesis; *rif*, rifampin sensitivity; *str*, streptomycin sensitivity; *trp*, tryptophan synthesis.

[c] Phenotype symbols: Aer$^-$, unable to grow aerobically; As^R, resistant to arsenate; P630, accumulate a BChl precursor with 630-nm absorption peak; P670, accumulate a BChl precursor with 670-nm absorption peak; PS$^-$, unable to grow photosynthetically; Rif^R, rifampin resistant; Str^R, streptomycin resistant; Trp$^-$, tryptophan auxotrophy; POK^R, photooxidative killing resistant.

which used to be called compound Y (for yellow). "Green" mutants form yellowish green aerobic colonies.

The symbol *bch* is proposed to designate those loci involved in BChl biosynthesis. Two groups of mutants of this type are represented in the present study, both of which are unable to synthesize functional BChl (BChl$^-$) and accumulate what appear to be precursors. These groups of mutants are named for the red-most absorption maximum of the accumulated compounds, as measured in sonic supernatants. Mutants accumulating compounds that absorb in the region between 630 and 635 nm are called P630, and those with peaks between 665 and 670 nm are called P670. Both types of mutant are incapable of photosynthetic growth. Similar mutants have been described previously, both for this species (2) and for *R. sphaeroides* (9).

Double mutants, carrying lesions that independently block carotenoid and BChl biosynthesis, were isolated by photooxidative killing (POK) of blue-green mutants. Cells unable to synthesize mature BChl are resistant to POK, and colonies of these double mutants take on characteristic colors depending on the BChl precursor accumulated. P670-Crt$^-$ mutants form greenish colonies, and P630-Crt$^-$ mutant colonies are yellowish.

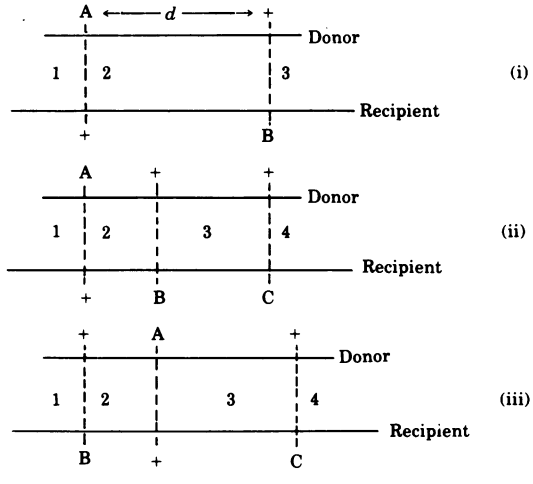
Media and growth conditions. Peptone-yeast extract medium (0.3% peptone [Difco] and 0.3% yeast extract [Difco] in deionized water) was used for growth of all cultures. It should be noted that some lots of yeast extract would support neither rapid growth nor gene transfer agent (GTA) production. Media for tryptophan auxotrophs were supplemented with 10 μg of L-tryptophan per ml. Photosynthetic growth of liquid cultures occurred in completely filled screw-capped tubes incubated at 35 C and illuminated at a light intensity sufficient to promote maximal growth rates (~40 W/m^2; measured with a YSI-Kettering model 65A radiometer through a Corning colored-glass filter CS#7-69; this filter transmits only the near-infrared region of the spectrum, in which the major absorption bands of in situ BChl lie). Photosynthetic cultures of blue-green mutants were always preincubated anaerobically in the dark for 4 to 5 h with 0.5 mg of sodium ascorbate per ml added to the medium to remove oxygen before illumination, thus avoiding photooxidative damage. Furthermore, blue-green mutant strains were maintained as aerobic stocks until photosynthetic growth was required, at which time heavy inocula of fresh aerobic transfers were used to start photosynthetic cultures. This procedure avoided accumulation of Crt$^+$ revertants, which grow faster than blue-green mutants under photosynthetic conditions. Photosynthetic incubations of seeded agar plates

were carried out in transparent anaerobic jars (BioQuest) incubated in a 30 C room and illuminated at an approximate intensity of 10 W/m² (measured as described above). Aerobic (dark) incubations were at 35 C, either in total darkness (standing liquid and plated cultures) or in ambient fluorescent lighting (agitated liquid cultures).

Gene transfer procedures. Gene transfers were performed as described previously (13), with the following modifications. A cell-free filtrate of the donor culture was mixed with recipient cells that had been pelleted and resuspended in buffer (13). The mixture was incubated at 35 C for 30 to 60 min under aerobic (dark) conditions to allow uptake of GTA. To select for the transfer of antibiotic resistance markers, samples of this mixture were plated in peptone-yeast extract medium containing 0.6% agar and incubated 3 to 4 h at 35 C to allow for phenotypic expression. Plates were then overlaid with soft agar containing the antibiotic. When selection for transferants was to be by POK, cultures were spread on the surface of agar plates immediately after the uptake of GTA. The spread plates were mounted on supports that fit inside the GasPak anaerobic jars, holding the plates so that the surface of the agar was perpendicular to the light path. Anaerobic jars were incubated in the dark at 30 C until most of the oxygen was consumed (about 90 min) and then illuminated 3.5 to 4 h under the conditions described above, thus allowing for phenotypic expression during photosynthetic growth. We are indebted to Paul Weaver for pointing out that *R. capsulata* blue-green mutants are only sensitive to POK for a short period after exposure to O_2. Therefore, immediately after anaerobic phenotypic expression, each plate was exposed for 15 min to the light from a 150-W flood lamp. A 10-cm wide water bath was used as a heat trap between the lamp and the plate. The light intensity at the surface of the plate, as measured through the CS#7-69 filter described above, was approximately 100 W/m². Plates were then incubated at 35 C, either aerobically or photosynthetically, until colonies were large enough for their carotenoid contents to be easily recognizable (generally 2 days). This procedure for POK left surviving colony-forming units at a frequency of about 10^{-5} from control blue-green cultures not treated with GTA. The actual frequency of survivors varied greatly with the blue-green strain used, both with respect to the quantity of survivors and their phenotypes. Surviving phenotypes (POK^R)included various revertants and partial revertants which had regained the ability to synthesize colored carotenoids, nonphotosynthetic mutants which had lost BChl synthetic ability, and the apparently unchanged blue-green starting phenotype. Photosynthetic outgrowth after POK favors colony formation by recombinants and revertants that have regained carotenoids, and induces more extensive pigment production. Thus, it was the method of choice (except for crosses with "yellow" donors, since the "yellow" phenotype cannot be distinguished from wild type in photosynthetic colonies). Control experiments demonstrated that the frequency of cotransfer of carotenoid markers is essentially independent of both the time of phenotypic expression and the time of exposure to POK conditions.

For crosses involving nonphotosynthetic recipients, recipient cultures were grown aerobically with agitation, but otherwise gene transfers were performed as usual. Samples of the culture, after GTA uptake, were plated in soft agar, allowed 3 to 4 h of aerobic (dark) incubation for phenotypic expression, and then incubated for 2 days anaerobically in light for selection of photosynthetically competent recombinants.

Analysis of genetic data. The gene transfer system was used to perform crosses involving one, two, or three markers. The results of these recombination tests were analyzed in two different ways, both of which arise from classical genetics of merozygotic crosses. The following diagrams will be referred to in explaining our application of these analyses.



Cotransfer analysis: two-point crosses. As diagrammed in i, the mutation carried by the donor GTA (A) causes the accumulation of "yellow" or "green" carotenoids. The recipient carries a mutation (B) that permits selection against the recipient. B may result in a block in BChl biosynthesis and thus a nonphotosynthetic phenotype. Alternatively, B may result in an inability to synthesize colored carotenoids, the blue-green phenotype, thus rendering the recipient sensitive to POK. The treatments that are applied after gene transfer each formally select for one recombination in region 3 plus one in either region 1 or 2. (For the purpose of this analysis, we have assumed that recombinants arise as the result of pairs of recombination events, ignoring odd numbers of exchanges because of the merozygotic nature of the cross and presuming even numbers of exchanges greater than two to be relatively rare.) If the second exchange occurs in region 2, the resulting transferants will accumulate wild-type carotenoids. However, if the second exchange occurs in region 1, carotenoids typical of the donor are accumulated, and we term the event a cotransfer. The frequency of cotransfer (ϕ) is assumed to be a function of the distance between A and B. The function may be a

complex one since, as the length of region 2 increases, several processes may serve to decrease the cotransfer of A and the wild-type site corresponding to B. For example, since the incoming donor DNA is limited in size, we expect the probability of any given donor molecule to carry both points to decrease as the distance between them increases. Furthermore, as the distance between A and B increases, the probability of exchange in region 2, a cotransfer-destroying event, increases. The cotransfer frequency is determined experimentally by dividing the number of colonies with the cotransfer phenotype by the total number of colonies with the single-transfer phenotype. Since only GTA-induced phenotypes are to be considered, corrections for spontaneous changes must sometimes be applied.

We have empirically arrived at the following map function for relating cotransfer frequency (ϕ) to the map distance (d) between markers A and B:

$$\phi = (1 - d)^2, \text{ or } d = 1 - \phi^{1/2}$$

Map distances generated by this function from cotransfer data fulfill the criterion used to select the function, namely, additivity. A function of this form is derived from basic considerations in the Appendix.

Cotransfer analysis: three-point crosses. Only one type of three-point cross is considered in the present work. The donor carries a single mutation (A) causing the accumulation of "green" or "yellow" carotenoids, and the recipient always carries two mutations, one that blocks colored carotenoid synthesis (B) and one that blocks BChl synthesis (C). The recombinant progeny resulting from gene transfer are selected for the ability to photosynthesize, and each PS^+ transferant is classified with regard to carotenoid content. If the gene order is A-B-C, as shown in diagram ii, then recombinations in region 4 plus region 3 yield blue-green transferants, recombinations in regions 4 and 2 yield the wild type, and recombinations in regions 4 and 1 yield colonies pigmented like the donor. If mutation A is closer to C than is mutation B, as shown in diagram iii, recombinations in region 4 plus region 2 or region 3 lead to blue-green transferants, and recombinations in region 4 plus region 1 lead to donor-type pigmentation. Wild-type transferants would be absent or very rare if the latter gene order is correct, since the generation of a wild type would require one recombination in each of regions 4, 3, 2, and 1. Thus, by determining the frequency of wild-type transferants, an order may be assigned and then, by analyzing the frequency of each carotenoid type, map distances may be computed for the A to C and B to C linkages.

Ratio test. The distance between two markers causing the same phenotype may be estimated in certain cases by the following adaptation of this well-established genetic test (6). Consider the cross represented by diagram i, when both markers A and B cause the blue-green phenotype. When recombinations occur in regions 2 and 3, wild-type recombinants are generated. However, if regions 1 and 3 are involved, a potential survivor of POK will be lost, because the lesion from the donor will be included in a cotransfer event. The frequency of these events may be calculated if we can estimate the number of "missing" recombinants. Toward this end we estimate an expected number of recombinants by comparing the test cross (diagram i) to a "one-point" cross, in which the particular recipient is treated with GTA carrying the wild-type region for carotenoid production. These two crosses are "compared" by reference to an unlinked marker that is the same in each cross, e.g., the *rif-10* mutation which causes rifampin resistance (Rif^R). If we let the subscript 1 stand for the results of the one-point cross and let 2 stand for the two-point cross, then:

$$Crt_2^+ \text{ (expected)} = Rif_2^R \times \frac{Crt_1^+}{Rif_1^R}$$

and

$$Crt_2^+ \text{ (missing)} = Rif_2^R \times \frac{Crt_1^+}{Rif_1^R} - Crt_2^+ \text{ (counted)}$$

Since

$$\phi = \frac{Crt_2^+ \text{ (missing)}}{Crt_2^+ \text{ (expected)}},$$

then

$$\phi = 1 - \frac{Crt_2^+ \text{ (counted)}}{Rif_2^R} \Big/ \frac{Crt_1^+}{Rif_1^R}$$

Cotransfer frequencies calculated in this way are subject to greater statistical variation than are frequencies determined by directly counting cotransfer events. The method of Kimball (7) was used to calculate a 95% confidence interval for the Crt^+/Rif^R ratios, and then the upper and lower limits were used to compute the largest interval for the cotransfer frequency. It should be noted that the Kimball method ignores all sources of error other than those due to statistical variation in sampling, and thus the confidence limits are conservative.

RESULTS

Crosses between yellow and blue-green mutants. When certain blue-green strains are treated with GTA from yellow strains, two types of POK^R recombinants are recovered: wild type and yellow. As discussed above, the yellow transferants arise from cotransfer events, and the frequency of cotransfer may be used to compute the map distance between the markers. From the data given in Table 2, it is apparent that all of the mutations that cause the yellow phenotype are linked to the blue-green-causing lesion in strain W4. The cluster of mutations that are about 20% cotransferable with that lesion is designated the *crtA* locus. (Since *cis-trans* complementation tests are not now possible in this organism, we cannot be certain that only one cistron is represented, but to retain a standard nomenclature for this genetic system clusters of markers giving the

same phenotype will be named as cistrons.) The crosses shown in the last three lines of Table 2 demonstrate that some mutations causing the blue-green phenotype are not closely linked to the *crtA* locus. The locus defined by the cluster of blue-green-causing mutations that are about 13% cotransferable with *crtA901* (*crtB4*, *crtB348* and *crtB16*) is designated *crtB*. The absence of yellow recombinants from the wild type (BB101) × W4 cross indicates that the yellow phenotype must be conferred by the donor GTA, and thus the yellow phenotype is not "present but masked" in the genome of the blue-green recipient.

Limits of GTA-mediated mapping. The frequency of simultaneous independent transfer events was measured previously for the *trpA20* and *rif-10* markers (13), and in the present experiments we routinely measured the frequency of Rif^R-Crt^+ (wild-type carotenoids) double events in crosses between Rif^S, blue-green recipients, and Rif^R,Crt^+ GTA. In all trials the frequency of simultaneous, independent events per total recipient colony-forming units was never greater than 10^{-8}. The highest frequency of single gene transfers per recipient that we have observed is about 4×10^{-4}. Since cotransfer analysis involves selection for one event, unlinked markers might give rise to apparent cotransfer frequencies as high as 4×10^{-4} (0.98 map unit). We observed several apparent cotransfer frequencies of this magnitude, but to determine if they represent actual linkages studies of cotransfer frequency as a function of GTA concentration would have to be done.

Crosses between blue-green mutants. To determine the number of loci carrying blue-green-causing mutations, crosses were performed between pairs of blue-green mutants. The *rif-10* marker was introduced into each strain by GTA, and the resulting Rif^R derivatives were used as donors for the crosses listed in Table 3. The numbers of Crt^+ transferants and Rif^R transferants were each determined, and the ratio test analysis was performed as described in Materials and Methods. All blue-green-causing mutations fall into two clusters by this test. One of these clusters contains the markers of *crtB*, and the other contains all of the remaining mutations of this class. The latter locus is termed *crtE*. The distance between *crtB4* and *crtB16* calculated from ratio test data (0.05 to 0.08 map unit) is in good agreement with the same distance determined as the difference between the appropriate distances measured by two-point crosses (0.05 map unit; data of Table 2).

Crosses between green and blue-green mutants. All mutations giving rise to green phenotypes show linkage to all *crtB* and *crtE* markers. These mutations fall into two clusters, *crtC* and *crtD*. Markers in the *crtC* locus map between 0.39 and 0.56 map unit from *crtB4*, whereas markers in the *crtD* locus map between 0.77 and 0.90 map unit from the same marker. The linkage relationships between the

TABLE 2. *Crosses between yellow donors and blue-green recipients*

Strain		*crt* markers	No. of transferants counted[a]			Cotransfer frequency[b]	Map distance[b]
Donor	Recipient	Donor × recipient	Red[c]	Bkg[d]	Yellow[e]		
BB101	W4	+ × *B4*	9,480	3	0	0	–[f]
BY711	W4	*A71* × *B4*	798	33	267	0.251	0.50
B301	W4	*A301* × *B4*	1,480	58	469	0.248	0.50
Y87	W4	*A87* × *B4*	345	1	97	0.220	0.53
SB35	W4	*A235* × *B4*	141	0	39	0.217	0.53
HH902	W4	*A902* × *B4*	918	1	220	0.193	0.56
SB7	W4	*A207* × *B4*	865	2	188	0.179	0.58
SB3	W4	*A203* × *B4*	120	2	19	0.139	0.63
HH901	W4	*A901* × *B4*	359	0	58	0.139	0.63
HH901	Y16	*A901* × *B16*	453	0	52	0.103	0.68
HH901	MB1048	*A901* × *B348*	921	332	112	0.160	0.60
HH901	W8	*A901* × *E8*	485	4	0	0	–
BY711	W6	*A71* × *E6*	5,104	0	0	0	–
SB7	W6	*A207* × *E6*	11,121	0	0	0	–

[a] Sample size is constant for red, Bkg, and yellow counts; selection was for POK^R (capacity to synthesize colored carotenoids).

[b] Calculated as described in the text.

[c] The number of colonies with red carotenoids; GTA-treated sample.

[d] The number of colonies with red carotenoids; sample not treated with GTA; no donor carotenoids (yellow) have ever been observed in these controls.

[e] The number of colonies with yellow carotenoids; GTA-treated sample.

[f] Column heading is not applicable.

TABLE 3. *Crosses between blue-green strains*

Strain		*crt* markers	Crt$^+$/Rif^R [a]	95% Confidence interval[b]	Cotransfer frequency[c]	Map distance[d]
Donor	Recipient	Donor × recipient				
BB101	W4	+ × *B4*	0.85	0.76–0.94	–[e]	–
BW5	W4	*E5* × *B4*	0.88	0.77–0.99	0	–
BW7	W4	*E7* × *B4*	0.85	0.76–0.94	0	–
BY771	W4	*E77* × *B4*	0.82	0.70–0.95	0	–
BY161	W4	*B16* × *B4*	0.10	0.09–0.11	0.90–0.86	0.05–0.08
BW6	W6	*E6* × *E6*	(0/2648)[f]	–	–	–
BB101	W6	+ × *E6*	0.94	0.87–1.01	–	–
BY161	W6	*B16* × *E6*	0.85	0.75–0.96	(0.26–0)[g]	(0.49–1)[g]
BW5	W6	*E5* × *E6*	0.13	0.11–0.15	0.89–0.83	0.06–0.09
BY771	W6	*E77* × *E6*	0.11	0.10–0.12	0.91–0.86	0.05–0.07

[a] The ratio of the number of Crt$^+$ to Rif^R transferants; in each case more than 2,000 Rif^R colonies were counted; Crt$^+$ were counted after selection for POK^R.

[b] The 95% confidence interval for Crt$^+$/Rif^R was determined by the method of Kimball (7).

[c] The cotransfer frequency between the *crt* markers in each cross is calculated from the Crt$^+$/Rif^R ratios as described in the text. The interval is the largest consistent with the 95% confidence intervals from the preceding column.

[d] The distance in map units between the two *crt* markers in each cross is calculated from the cotransfer frequency as described in the text.

[e] Column heading is not applicable.

[f] No Crt$^+$ recombinants were detected in a sample containing 2,648 Rif^R recombinants.

[g] The sample mean in this experiment was outside the 95% confidence interval of the control cross (BB101 × W6); therefore the cotransfer frequency and map distance were computed, although values in this range clearly give rise to map distances of great uncertainty.

crtC and *crtD* genes and mutation *crtE6* are complementary to those just described for *crtB4*; i.e., markers in *crtC* lie between 0.35 and 0.56 and *crtD* markers lie between 0.13 and 0.25 map unit from *crtE6* (Table 4). The phenotypes conferred by *crtC* and *crtD* mutations are distinguishable upon careful examination, the former class giving rise to slightly yellower colonies than the latter. A preliminary separation of the carotenoids of one mutant from each class suggests that the *crtC* mutations cause the accumulation of significant quantities of only neurosporene, whereas mutants with a lesion in *crtD* accumulate neurosporene plus another carotenoid with a nonaene chromaphore. These phenotypic differences support the genetic data with respect to the existence of two separate loci giving rise to green mutants. The data in Table 5 show that each marker of clusters *crtB* and *crtE* cotransfers with marker *crtD223* at a frequency characteristic for its cluster, thus supporting their assignments based on the yellow × blue-green crosses. The only sequence for the *crt* genes that is compatible with the foregoing data is shown in Fig. 1. This order is supported by linkage studies between the green mutants and BChl$^-$ mutants.

Linkage between *crt* and *bch* genes. BChl$^-$ mutants were treated with GTA carrying each type of *crt* lesion, and selection for BChl$^+$ recombinants was performed as described in Materials and Methods. The only cotransfers observed were between mutations giving rise to the P670 and P630 phenotypes and the *crtD* gene markers. Since the mutations giving rise to these two BChl$^-$ phenotypes appear to be linked to *crtD*, but not *crtC* or *crtA*, the genes defined by P670-causing and P630-causing mutations are placed to the right of the carotenoid region on our map. These genes are designated *bchA* and *bchB*, respectively. In support of this location for the loci concerned with BChl biosynthesis, the data of Table 6 show that one mutation of the *bchA* gene, *bchA62*, is more closely linked to the right side of *crtD* (*crtD223*) than to the left side (*crtD39*). The other crosses of Table 6 are consistent with this gene order, but the weak linkage between *crtD* and *bchA* genes, together with the poorer recipient activity of aerobically grown cultures, hinders collection of sufficient data for confident ordering of most *crtD* markers by this means. On the other hand, the low recipient activity of BChl$^-$ mutants probably decreases the chances of observing double mutants (green,BChl$^+$) that result from simultaneous, independent gene transfers. Simultaneous, independent gene transfers for the *rif-10* and *bch* markers were observed in one cross reported in Table 6, although at a frequency much lower

TABLE 4. *Crosses between green donors and certain blue-green recipients*

Donor		Recipient strain (marker)								
		W4 (*B4*)				W6 (*E6*)				
Strain	*crt* marker	Transferants[a]		ϕ[b]	d_1[c]	Transferants[a]		ϕ[b]	d_2[c]	$d_1 + d_2$[d]
		Red[e]	Green[f]			Red[e]	Green[f]			
Y150	*D150*	363	4	0.011	0.90	408	1,267	0.76	0.13	1.03
SB23	*D223*	5,851	83	0.014	0.88	405	1,101	0.73	0.15	1.03
Y127	*D127*	—[g]	—	—	—	52	139	0.73	0.15	—
Y125	*D125*	—	—	—	—	27	61	0.69	0.17	—
SB38	*D238*	—	—	—	—	15	33	0.69	0.17	—
Y83	*D83*	227	9	0.038	0.81	186	310	0.64	0.20	1.01
SB37	*D237*	—	—	—	—	12	20	0.63	0.21	—
BY392	*D39*	8,310	121	0.014	0.88	413	670	0.62	0.21	1.09
Y361	*D361*	2,147	116	0.051	0.77	379	486	0.56	0.25	1.02
Y88	*C88*	95	35	0.27	0.48	85	62	0.42	0.35	0.83
R114	*C114*	—	—	—	—	39	23	0.37	0.39	—
Y129	*C129*	65	18	0.22	0.53	114	71	0.38	0.38	0.91
M112	*C112*	1,040	241	0.19	0.56	727	308	0.30	0.45	1.01
Y68	*C68*	—	—	—	—	40	15	0.27	0.48	—
SB9	*C209*	28	12	0.30	0.45	454	155	0.25	0.50	0.95
BY761	*C76*	608	213	0.26	0.49	1,663	504	0.23	0.52	1.01
Y59	*C59*	—	—	—	—	186	51	0.22	0.53	—
BY612	*C61*	211	80	0.27	0.48	484	109	0.21	0.54	1.02
SB36	*C236*	63	31	0.33	0.43	88	20	0.19	0.56	0.99
Y126	*C126*	33	19	0.37	0.39	104	25	0.19	0.56	0.95

[a] Selection was for POK^R.

[b] ϕ, Cotransfer frequency, calculated as described in the text.

[c] d_1, Map distance between the donor *crt* marker and *crtB4*; d_2, map distance between the donor *crt* marker and *crtE6*.

[d] The map distance between *crtB4* and *crtE6* calculated as the sum of the experimentally determined distances d_1 and d_2.

[e] The number of wild-type colonies counted; corrected for spontaneous background.

[f] The number of green-colored colonies counted in the same sample as the red; spontaneous background for this type is always zero.

[g] Values not determined.

TABLE 5. *Crosses between a green donor and various blue-green recipients*

Recipient strain	*crt* marker	Transferants[a]		ϕ[b]	d[b]
		Red[b]	Green[b]		
W5	*E5*	202	1,383	0.87	0.07
W7	*E7*	243	1,285	0.84	0.08
Y77	*E77*	22	98	0.81	0.10
W8	*E8*	25	81	0.76	0.13
Y90	*E90*	8	22	0.73	0.15
W6	*E6*	405	1,101	0.73	0.15
W9	*E9*	180	451	0.71	0.16
Y16	*B16*	1,261	26	0.020	0.86
W4	*B4*	5,851	83	0.014	0.88
MB1048	*B348*	1,467	13	0.0088	0.91

[a] The donor strain for all crosses was SB23; selection for POK^R.

[b] As in Table 4.

than that observed for green,BChl$^+$ transferants.

Linkage between the *crtE* and *bchA* loci can be demonstrated when blue-green,BChl$^-$ double mutants are used as recipients for wild-type GTA (Table 7). The map distances obtained with the *crtE6,bchA* double mutants suggest that *crtE* is indeed closer to *bchA* than is *crtD*, and the three-point crosses with *crtD*-marked GTA confirm this (see below). Two blue-green, P670 double mutants, carrying the *crtB4* lesion, fail to give rise to any Crt$^+$,BChl$^+$ cotransferants when treated with wild-type GTA. This result would be expected if *bch-165* and *bch-167*, the P670-causing lesions in question, map in *bchA*, since cotransfers between markers separated by more than 0.98 map unit are exceedingly infrequent and are difficult to distinguish from background.

Three-point crosses. The order of genes *crtD* and *crtE* relative to *bchA* can be tested by treating *crtE,bchA* double mutants with GTA from green mutants with a *crtD* lesion. Since transferants with wild-type carotenoids are more frequent than green transferants among

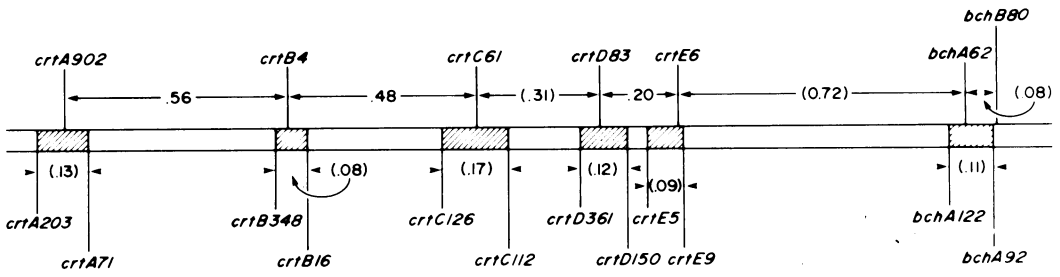


FIG. 1. *Genetic map of the region for carotenoid and BChl biosynthesis. The numbers above the map represent distances, in map units, between specific markers in each gene. The numbers below the map give the distances between the (current) terminal markers in each gene, thus providing estimates of the minimum length of each gene. Distances were calculated from cotransfer frequencies as described in the text. Distances obtained by subtraction are given in parentheses.*

TABLE 6. *Crosses between donors with altered carotenoids and recipients with altered BChl*

Donor strain	Recipient strain	Relevant markers	PS⁺ transferants[a]			ϕ[b]	d[b]
			Crt⁺[c]	Crt^D[d]	Rif^R[e]		
Y127	Y62	*crtD127* × *bchA62*	206	4	—[f]	0.0190	0.86
SB23	Y62	*crtD223* × *bchA62*	23,270	422	3	0.0178	0.87
Y39	Y62	*crtD39* × *bchA62*	2,417	34	—	0.0140	0.88
Y150	Y62	*crtD150* × *bchA62*	364	4	—	0.0109	0.90
SB38	Y62	*crtD238* × *bchA62*	441	4	—	0.0090	0.91
Y361	Y62	*crtD361* × *bchA62*	379	3	—	0.0079	0.91
Y83	Y62	*crtD83* × *bchA62*	642	3	—	0.0047	0.93
W6	Y62	*crtE6* × *bchA62*	1,291	0	—	—	—
SB36	Y62	*crtC236* × *bchA62*	1,926	0	—	—	—
SB9	Y62	*crtC209* × *bchA62*	3,552	0	—	—	—
Y59	Y62	*crtC59* × *bchA62*	3,876	0	—	—	—
Y71	Y62	*crtA71* × *bchA62*	1,135	0	—	—	—
SB23	SB21	*crtD223* × *bchA221*	1,350	25	—	0.0182	0.87
Y39	SB21	*crtD39* × *bchA221*	1,739	10	—	0.0057	0.92
Y59	SB21	*crtC59* × *bchA221*	1,086	0	—	—	—
SB7	SB21	*crtA7* × *bchA221*	3,884	0	—	—	—
SB23	Y34	*crtD223* × *bchA34*	1,536	10	—	0.0065	0.92
SB23	Y92	*crtD223* × *bchA92*	1,215	4	—	0.0033	0.94
SB23	Y80	*crtD223* × *bchB80*	2,342	5	0	0.0021	0.95

[a] Selection was for photosynthetic growth.
[b] As in Table 4.
[c] Number of colonies with carotenoids like the wild type.
[d] Number of colonies with carotenoids like the donor.
[e] Number of rifampin-resistant PS⁺ colonies.
[f] Values not determined.

BChl⁺ recombinants, the *crtE* gene must lie between *crtD* and *bchA* (Table 7). This finding is consistent with the order of markers established by analyzing map distances from two-point crosses.

Additivity of map distances. The choice of the function for transforming cotransfer frequencies into map distances rests on the conventional assumption that genetic markers are arranged in a linear array, and thus the distances between them should be additive. The ability of the function $\phi = (1 - d)^2$ to generate distances that are additive is demonstrated by the data in Tables 4 and 7. The distance between *crtB4* and *crtE6* can be obtained by adding the distances between a central marker and each of these two outside markers. Since all of the markers in the *crtC* and *crtD* loci lie between *crtB4* and *crtE6*, a wide range of central marker positions is available, and the constancy of the calculated distance (Table 4, column 11) demonstrates additivity. The three-point crosses (Table 7) provide another test of additivity. The data show clearly that the distance between the outside markers (d_3), here measured directly, equals the sum of the distances between each outside marker and a central marker ($d_1 + d_2$).

TABLE 7. *Crosses between green donors and blue-green, BChl⁻ recipients*

Donor strain	Recipient strain	Relevant markers	PS⁺ transferants[a]			d_1[b]	d_2[c]	d_3[d]	$d_1 + d_2$[e]
			Crt⁺[f]	Crt^D[g]	Crt^R[h]				
SB1003	Y121	*crt⁺ × crtE6, bchA121*	73	–[i]	1,041	–	0.74	–	–
SB23	Y121	*crtD223 × crtE6, bchA121*	175	46	5,117	0.15	0.80	0.91	0.95
Y39	Y121	*crtD39 × crtE6, bchA121*	36	9	1,095	0.21	0.80	0.91	1.01
Y150	Y121	*crtD150 × crtE6, bchA121*	658	84	15,913	0.13	0.79	0.93	0.92
SB1003	Y122	*crt⁺ × crtE6, bchA122*	190	–	1,835	–	0.69	–	–
SB23	Y122	*crtD223 × crtE6, bchA122*	80	12	830	0.15	0.67	0.89	0.82
SB1003	Y165	*crt⁺ × crtB4, bch-165*	0	–	1,079	–	≥1	–	–
SB23	Y165	*crtD223 × crtB4, bch-165*	0	0	830	0.88	≥1	≥1	–
SB1003	Y167	*crt⁺ × crtB4, bch-167*	0	–	1,612	–	≥1	–	–
SB23	Y167	*crtD223 × crtB4, bch-167*	0	0	1,182	0.88	≥1	≥1	–

[a] Selection was for photosynthetic growth.
[b] Map distance between the donor and recipient *crt* markers; data from Table 4.
[c] Map distance between the *crt* and *bch* markers of the recipient.
[d] Map distance between the *crt* marker of the donor and the *bch* of the recipient.
[e] Sum of the map distances indicated (see text).
[f] Number of colonies with wild-type carotenoids.
[g] Number of colonies with carotenoids like the donor.
[h] Number of colonies with carotenoids like the recipient.
[i] Column heading is not applicable.

DISCUSSION

The foregoing results demonstrate that the gene transfer system of *R. capsulata* is a useful tool for mapping chromosomal regions that are about the size of an operon. The genetic map (Fig. 1) summarizes the linkage data presented in this report. The map order and distances derived from this genetic system seem consistent and unambiguous. Marker-specific transfer efficiency differences are not large and do not strongly affect mapping. Interference phenomena were not observed, although the use of an empirical map function might compensate for what would otherwise be termed interference. Only three mutant strains that gave unusual recombination values were discovered. Each of these strains, which are not included in this report, behaved as if it carried multisite mutations. This would not be surprising, since nitrosoguanidine has been shown to cause clustered mutagenic hits (5). Taking the map distance between the extreme markers of each cluster as an estimate of the dimensions of a gene in map units, 1 map unit would correspond to about 5 to 10 genes, and the entire mapped region is probably less than 1% of the genome.

The map function that serves to transform cotransfer data from this genetic system into map distances is similar in form to a mapping function derived by Wu (14) for transduction:

$$\text{cotransduction frequency} = \left(1 - \frac{\text{distance between two markers}}{\text{length of transducing particle}}\right)^3$$

However, Wu's function does not fit the data generated by the *R. capsulata* gene transfer system. His derivation assumes donor fragments of nearly constant length from which various regions may be incorporated into the recipient chromosome by pairs of crossover events. If one assumes, instead, that the region incorporated is bounded by a crossover event on one side and an end of the donor fragment on the other, the predicted map function would be essentially the same as the empirical function:

$$\text{cotransfer frequency} = \left(1 - \frac{\text{distance between two markers}}{\text{length of gene transfer agent}}\right)^2$$

(see Appendix)

This suggests that the map distance calculated using the empirical mapping function is actually the ratio of intermarker distance to a constant, i.e., the length of GTA DNA. These genetic considerations lead us to expect GTA DNA to code for 5 to 10 genes, an expectation that is consistant with the mass of GTA DNA estimated from the physical characteristics of the GTA particle (13). Since the length of the DNA molecule carried by GTA can be independently determined (M. Solioz and B. Marrs, manuscript in preparation), map distances may be converted to nucleotide distances if this model is appropriate. Although the applicability of this model cannot be tested at this time, no substantially different alternative that fits the data has occurred to us.

Crosses between *crtE*-bearing GTA and *bchA*-marked recipients fail to give rise to blue-green recombinants (see Table 6; W6 × Y62 → no blue-green transferants out of 100 expected).

Markers from these same loci do give blue-green recombinants when they enter the cross in the *cis* position in the recipient (Table 7). These results appear to be due to a requirement for a second mutation, unlinked to the *crtE* gene, in order to obtain strains capable of photosynthetic growth in the presence of a *crtE* lesion. If this is correct, it implies that the phenotype of this typical blue-green strain of *R. capsulata* may not be a simple result of the absence of colored carotenoids, but may include compensatory alterations in the residual photosystem. This hypothesis is currently under investigation. The discovery that some genes for the synthesis of BChl are linked to carotenoid synthetic genes suggests a transcriptional basis for the coordinate response of these two photopigments to regulatory stimuli. The map location of mutations affecting the regulation of photopigment synthesis should prove interesting with regard to this hypothesis.

APPENDIX

Models for Recombination in Merozygotic Systems

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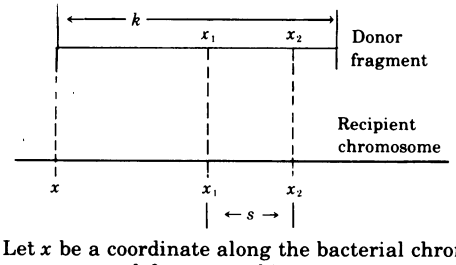
As noted above, transformation of experimental values of the cotransfer frequency (ϕ), obtained using the gene transfer system of *R. capsulata*, to map distances (d) that are properly additive is effected by the relation:

$$\phi = (1 - d)^2 \tag{1}$$

Since this relation is somewhat unusual, it is of interest to try to construct a model of genetic recombination that generates it. In this appendix, three models of recombination are examined, one of which generates the desired relation.



Let x be a coordinate along the bacterial chromosome, measured from an arbitrary origin. Assume that fragments of length k are cut from the chromosome at random such that the probability of occurrence of a fragment with a left terminus in the interval x to $x + dx$ is νdx, where ν is the probability of breakage per unit length and independent of x. Assume that the fragments may be transferred from donor to recipient cells, wherein they may replace homologous portions of the genome via a pair of crossover events leading to recombination. Consider loci at x_1 and x_2 separated by distance s, equal to $x_2 - x_1$, where $s < k$. The probability of more than two crossover events in the length k is ignored.

In model A, the probability of a crossover event at any point in an interval of the genome is assumed to be proportional to the length of the interval. Thus, the probability of a crossover in the interval (x, x_1) is $\mu(x_1 - x)$ and the probability of a crossover in the interval $(x_1, x + k)$ is $\mu(x + k - x_1)$, where μ is a proportionality constant independent of x. x is the coordinate of the left terminus of the fragment. Assuming that crossover probabilities are independent, we have that the probability of incorporation of a locus at x_1 is:

$$p_1^A = \int_{x_1 - k}^{x_1} \nu\mu^2(x_1 - x)(x + k - x_1)dx = \frac{\mu^2\nu k^3}{6} \tag{2}$$

Similarly, the probability of incorporation of loci at x_1 and x_2 is:

$$p_{12}^A = \int_{x_2 - k}^{x_1} \mu\nu^2(x_1 - x)(x + k - x_2)dx = \frac{\mu^2\nu}{6}(k - s)^3 \tag{3}$$

The cotransfer frequency ϕ^A for model A is given by:

$$\phi^A = \frac{p_{12}^A}{p_1^A} = (1 - s/k)^3 \tag{4}$$

The ratio s/k is a map distance d, in units of k.

In model B, the probability of one crossover event in an interval is assumed to be proportional to the interval length, but a second crossover event is assumed to take place only at, or very near, a terminus of the fragment, with probability ϵ, where ϵ is independent of x. The resulting expressions are the same no matter which end is chosen for the site of the terminal crossover; so in this case we arbitrarily choose the right end. Thus, the probability of a crossover in the interval (x, x_1) is assumed to be $\mu(x_1 - x)$ and the probability of a crossover in the interval $(x_1, x + k)$ is assumed to be ϵ. This leads to a probability for the incorporation of locus x_1 of:

$$p_1^B = \int_{x_1 - k}^{x_1} \nu\mu\epsilon(x_1 - x)dx = \frac{\nu\mu\epsilon k^2}{2} \tag{5}$$

Similarly, the probability of incorporation of loci at x_1 and x_2 is:

$$p_{12}^B = \int_{x_2 - k}^{x_1} \nu\mu\epsilon(x_1 - x)dx = \frac{\nu\mu\epsilon}{2}(k - s)^2 \tag{6}$$

The co-transfer frequency ϕ^B is then:

$$\phi^B = \frac{p_{12}^B}{p_1^B} = (1 - s/k)^2 \tag{7}$$

In model C, it is assumed that the entire fragment is inserted in all cases, because crossover may only occur at its ends, with probabilities of ϵ_L and ϵ_R for

the left and right ends, respectively. Then, for incorporation of locus x_1:

$$p_1^C = \int_{x_1 - k}^{x_1} \nu\epsilon_L\epsilon_R dx = \nu\epsilon_L\epsilon_R k \quad (8)$$

For incorporation of loci x_1 and x_2:

$$p_{12}^C = \int_{x_2 - k}^{x_1} \nu\epsilon_L\epsilon_R dx = \nu\epsilon_L\epsilon_R(k - s) \quad (9)$$

Therefore, the cotransfer frequency ϕ^C is given by:

$$\phi^C = \frac{p_{12}^C}{p_1^C} = (1 - s/k) \quad (10)$$

Models A and C are presented for comparison. Model A was constructed and discussed by Wu (14) and is considered appropriate to some generalized transducing systems. Model B is the case of particular interest here, as it generates equation 1.

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